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GREEN HEMOPROTEIN OF ERYTHROCYTES: METHEMOGLOBIN SUPEROXIDE TRANSFERASE

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Influences of base (pH 10), heat (50° C), microwave radiation (2450 MHz, 103 ± 4 W/kg), and hydrogen peroxide (5.6 mM) generated by glucose oxidase on oxidation of human oxyhemoglobin to methemoglobin were examined. Conversion of oxyhemoglobin to methemoglobin was followed by the difference in absorbancy of 540 or 542 nm and 576 nm wavelength light versus time. Fresh basic hemolysates auto-oxidized on heating with a zero order rate constant, implying that hemoglobin or another protein saturated with oxyhemoglobin catalyzed the oxidation. Simultaneous microwave irradiation inhibited thermally induced auto-oxidation on the average by 28.6%. However, there was great variability among samples and a decrease in auto-oxidation with aging of individual samples. The auto-oxidation rate was independent of initial oxyhemoglobin concentration. Oxidation of partially purified oxyhemoglobin by hydrogen peroxide was not influenced by microwave irradiation. Adding green hemoprotein isolated from human erythrocytes to the oxyhemoglobin/glucose oxidase reaction mixture yielded absorption spectra (500-600 nm) that were a combination of oxyhemoglobin, deoxyhemoglobin, and methemoglobin spectra. Green hemoprotein was labile in hemolysates but stable in a partially purified ferric form. These results imply that thermally unstable reduced green hemoprotein can reverse oxidation of oxyhemoglobin by hydrogen peroxide and could mediate the thermally induced and microwave inhibited auto-oxidation of oxyhemoglobin.

INTRODUCTION

Our previous work revealed that heating erythrocytes labeled with luminol yielded active oxygen metabolites[1,2] and that heating unlabeled erythrocytes above 42°C led to increased spontaneous hemolysis which was enhanced by a thiol reactive reagent (1-chloro-2,4-dinitrobenzene) and peroxidase[3]. The activation energy for the oxidation of the luminescent label on erythrocytes is 18.5 kJ/mole for temperatures 29°C and below and 53 kJ/mole for temperatures above 29°C.[2] The high activation energy process was inhibited by 2450 MHz (91 W/kg) microwave radiation.[2] However, the activation energy for the high energy oxidation was still considerably less than the 134 kJ/mole reported as the activation energy for the auto-oxidation of oxyhemoglobin.[4] Thermally induced hemolysis was also inhibited by microwave radiation (2450 MHz, 3,333 pulses per second, duty factor of 0.02, and average specific absorption rate of 0.4 W/kg), but only at 42°C.[3] These data led us to hypothesize that a protein other than hemoglobin itself was catalyzing oxyhemoglobin auto-oxidation.

A potential catalyst for the auto-oxidation of oxyhemoglobin is green hemoprotein (GHP), a single protein or family of heme proteins.[6] Its heme is completely lost by auto-oxidation at 4°C to −20°C within 24 hours when stored in hemolysates.[5,6] The protein can be preserved by freezing hemolysates to −60°C or less[6] and by maintaining the GHP in buffers containing dithiothreitol.[7] Purification decreases the

oxygen sensitivity of GHP so that it can be stored at −20°C indefinitely.[7] GHP is most unstable at its isoelectric point between pH 5 and 6.[7] The heme is readily bleached by dithionite and hydrogen peroxide, and reduction of the heme followed by addition of oxygen to yield superoxide leads to about a 20% loss of heme on each redox cycle [8] The rate constant for the auto-oxidation (release of superoxide) from bovine erythrocyte GHP is reported to be 501 sec^{-1} at 5°C.[8]

In this study, we first examined thermally induced auto-oxidation in fresh human hemolysates in basic (pH 10) buffer to minimize spontaneous auto-oxidation at 50°C with and without microwave radiation. Then, we partially purified oxyhemoglobin and GHP and examined the auto-oxidation of the oxyhemoglobin with glucose oxidase (GO, EC 1.1.3.4) and glucose at pH 7.4 with and without GHP. The purpose of these experiments was to determine if a thermolabile form of GHP could play a role in the auto-oxidation of oxyhemoglobin.

MATERIALS AND METHODS

Human red blood cells were obtained fresh daily from surplus clinical material, pooled, and hemolyzed in equal parts of distilled water to provide a source of oxyhemoglobin and green hemoprotein. Glucose oxidase (GO, EC 1.1.3.4), D-glucose, buffer salts, sodium hydrosulfite, column materials, and dithiothreitol were obtained from Sigma Chemicals (St. Louis, Missouri).

Fresh hemolysates of human blood cells were diluted 1:50 (total volume = 4.2 ml) in pH 10.0 buffer containing 0.09M sodium carbonate, 0.01M sodium phosphate, and 0.15M sodium chloride, and circulated (3.1 ml/min) through a 1-ml sample holder in a circularly polarized waveguide. The sample temperature was held at 50°C for 20 min by microwave radiation and/or forced hot air. The exposure system was the same as one described previously and with the same microwave absorption characteristics.[3,9] The only modification was the recycling of the solution by a peristaltic pump between the sample holder and a flow cell in a rapid scan diode

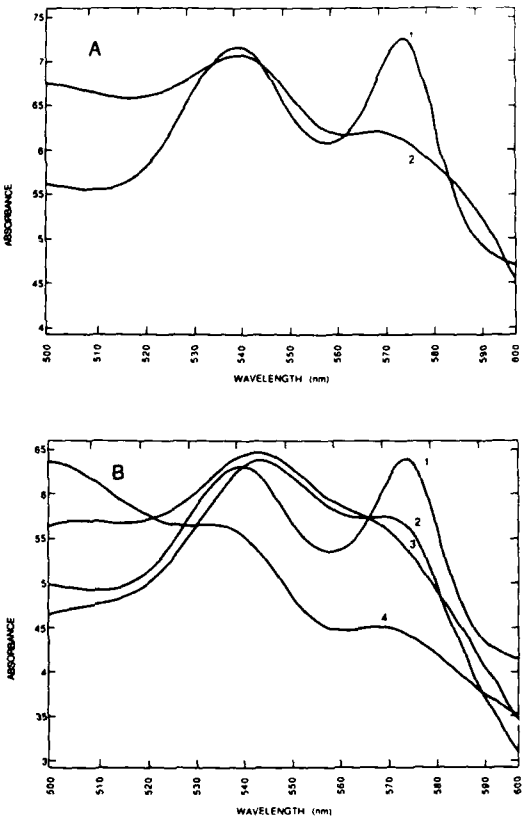

FIGURE 1. Absorbance spectra of human hemoglobin. Oxyhemoglobin was prepared by removal of anionic protein with DEAE-cellulose from 1:50 dilution fresh human hemolysates. Figure 1A, 100 μl 2 mg/ml (44 Sigma units) glucose oxidase were added to 1 ml of oxyhemoglobin in 0.01M potassium phosphate, pH 7.4, buffer; curve A1, oxyhemoglobin before addition of D-glucose; A2, methemoglobin formed during 21 min of incubation with glucose. Figure 1B, 100 μl green hemoprotein solution (mean absorbance at 404 nm = 0.3) was added to oxyhemoglobin that was then oxidized by glucose oxidase as in A. This green hemoprotein was prepared by elution from the DEAE-cellulose (used to partially purify the oxyhemoglobin) by linear pH (7.2 to 6.0) and ionic gradients (0.001 to 0.05M). Curve B1 was taken before addition of D-glucose; curve B2, 6 min later; B3, 9 min later; and B4, 21 min after addition of glucose. The sample was not mixed after initiation of the reaction.

array spectrophotometer (Hewlett Packard, 8451A). This same spectrophotometer was used to follow the oxidation of partially purified oxyhemoglobin and GHP by H_2O_2 produced from D-glucose and glucose oxidase (Figure 1).

Oxyhemoglobin was partially purified from the same hemolysates as the GHP by removing the GHP with DEAE-cellulose. Partial purification of GHP from fresh human hemolysates by anion and cation exchange chromatography[5,6] yielded a ferric anionic heme protein with a Soret peak at 404 nm that shifted to 416 nm upon reduction with dithiothreitol. The solutions we used had a 404 nm absorbance of about 0.3. This GHP rapidly and partially destroyed its heme content upon addition of a few crystals of sodium hydrosulfite with further destruction upon inclusion of 0.003% H_2O_2[8]. However, the addition of this concentration of H_2O_2 to the GHP without dithionite yielded no change in the Soret except for that which could be accounted for by dilution.

RESULTS AND DISCUSSION

Table I displays the results of 6 separate experiments and one repetition following incubation of the hemolysate at room temperature. Based on data from Table I, the mean rate constant for thermally driven auto-oxidation without microwave radiation was $3.99 \pm 0.79 \times 10^{-3}$ min^{-1} and with microwave radiation was $2.85 \pm 0.62 \times 10^{-3}$ min^{-1}, if experiment 5 is excluded. The range of differences between convection heated and microwave heated samples was, however, 17.2 to 46.2%. This variation and the decline in the difference from 25 to 3% when hemolysate was held at room temperature for 40 minutes before heating led to the conclusion that the auto-oxidation and microwave effect were mediated by a labile substance other than hemoglobin.

Heating the ferric form of GHP to 25, 30, 37, 42, 45, or 50°C for 30 min with or without microwave radiation at most led to loss of 3.4% of the Soret absorbance. Therefore, the ferric GHP was relatively insensitive to heat and microwave radiation.

Oxyhemoglobin converted to methemoglobin when treated with GO and D-glucose (1 mg/ml) at pH 7.4 (Figure 1A). Microwave radiation (2450 MHz, 103 W/kg) did not affect the rate of conversion at 25°C. When GHP was added to the oxyhemoglobin, glucose, and GO at a concentration that did

TABLE I. Microwave radiation (2450 MHz, 103 ± 4 W/kg) effects on the apparent zero order rate constants of thermally driven (50°C) auto-oxidation of human oxyhemoglobin in hemolysates at pH 10.

Experiment	Rate constants[a] ($\times 10^3$)		Percent inhibition
	Thermal	Microwave	
1	3.41	2.05	39.9
2	4.42	3.66	17.2
3	5.09	2.74	46.2
4	4.04	3.03	25.0
5[b]	3.61	3.50	3.0
6	4.15	3.36	19.0
7	2.83	2.28	19.4

[a] Rate constants are expressed as differences between absorbance at 540 or 542 nm and 576 nm per min based on spectra run every min for 20 min.

[b] Sample run from same hemolysate as in experiment 4, but after 40 min at room temperature (23°C); all other hemolysates were prepared fresh and briefly maintained at 4°C before being heated to 50°C.

not add to absorbance of hemoglobin between 500 and 600 nm, the conversion to methemoglobin seemed to be accelerated based on the similarity between the methemoglobin spectrum in Figure 1A and the apparent methemoglobin spectrum in Figure 1B. However, bubbling air through the sample with GHP partially recovered the double peak (540 and 576 nm) absorbance of oxyhemoglobin (Figure 2). Such a change was not observed when air was bubbled through samples without GHP. The methemoglobin-like spectrum seen with GHP reached a maximum absorbance in the range of 500 to 600 nm within 9 min of initiating the reaction by the addition of D-glucose (Figure 1B). By 21 min (Figure 1B), the spectrum was like that of methemoglobin in Figure 1A, but with a lower absorbance from 500 to 600 nm than the starting oxyhemoglobin. Thorough mixing of the GHP treated samples prevented the appearance of the 0 to 9 min spectrum. With mixing, as in the recycling waveguide system, the oxyhemoglobin containing GHP appeared to convert to methemoglobin much slower than that without GHP. Because of the much slower conversion of oxyhemoglobin to methemoglobin in the presence of GHP, a microwave effect could not be observed in the 20 min recycling time.

The methemoglobin-like spectrum can be thought of as a composite of oxyhemoglobin, deoxyhemoglobin, and methemoglobin spectra. Oxyhemoglobin has near equal peaks at 540 and 576 nm, deoxyhemoglobin has a peak at 555 nm, and methemoglobin (at pH 7.4) has peaks at 500 and 631 nm at a much lower absorbancy. Combining these spectra

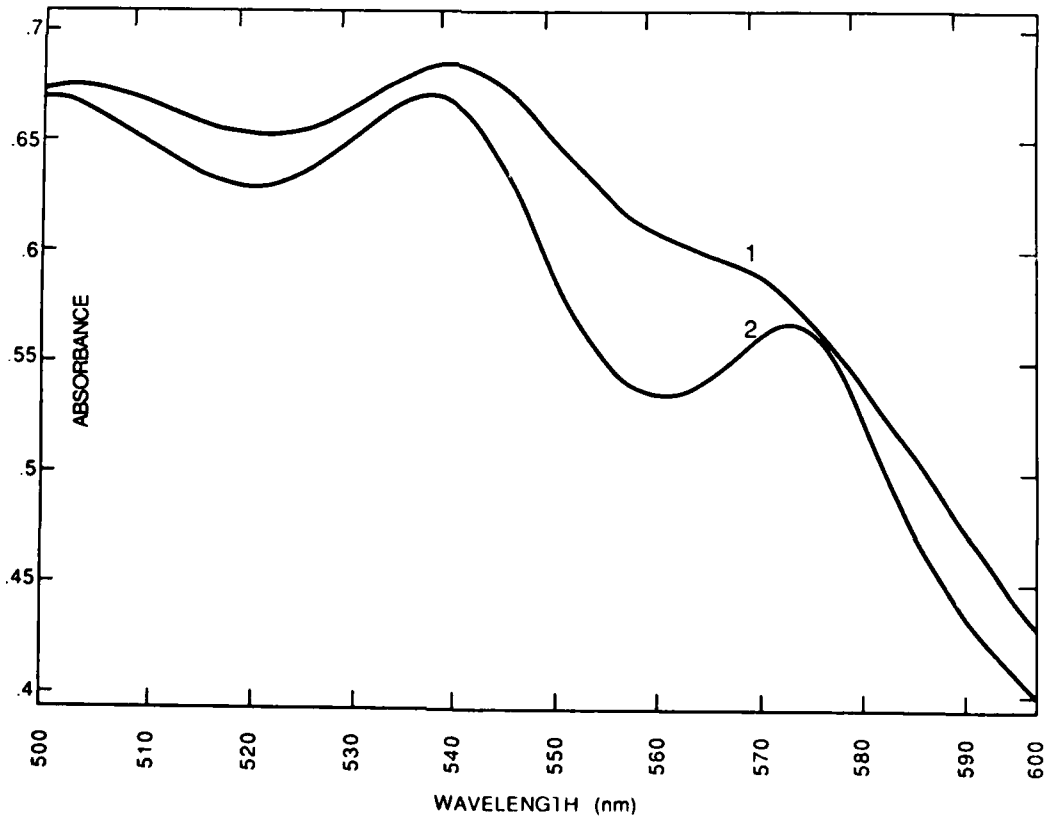

FIGURE 2. Absorbance spectra of human hemoglobin before (curve 1) and after (curve 2) bubbling air through a solution initially containing oxyhemoglobin, glucose oxidase, D-glucose, and green hemoprotein at concentrations described in Figure 1.

results in a broad absorbance peak between 540 and 570 nm with a slight depression at 560 (Figure 1B). Even under alkaline conditions (pH 9.5-10.0), the double peak (540 and 575 nm) of basic methemoglobin has a much lower extinction coefficient than oxyhemoglobin.[10]

Therefore, in the presence of GHP, GO, and 5.6 mM D-glucose, oxyhemoglobin is converted within 9 min to a mixture of oxyhemoglobin, deoxyhemoglobin, and methemoglobin with eventual complete conversion to methemoglobin. GHP must be converting at least some of the methemoglobin to oxyhemoglobin which deoxygenates as the GO uses oxygen to oxidize D-glucose to D-gluconic acid and H_2O_2. The process continues until all the oxygen or glucose is depleted in the solution or all the hemoglobin is converted to methemoglobin. The reactions can be summarized by the following scheme (where RH is a reductant such as thiol, unsaturated fatty acid, NADH, NADPH, or protein, MetHb is methemoglobin, and HbO_2 is oxyhemoglobin):

$$(1)\quad GHP + H_2O_2 + RH \rightarrow GHP(\cdot OH) + H_2O + R\cdot$$

$$(2)\quad GHP(\cdot OH) + H_2O_2 \longleftrightarrow GHP(\cdot O_2^-) + H_2O + H^+$$

$$(3)\quad GHP + R\cdot + O_2 \rightarrow GHP(\cdot O_2^-) + R^+$$

$$(4)\quad GHP(\cdot O_2^-) + MetHb \longleftrightarrow GHP + HbO_2$$

Although reactions (2) and (4) are reversible, the release of superoxide ($\cdot O_2^-$) from GHP is kinetically and thermodynamically favored and the transfer and binding of superoxide to methemoglobin is also favored because of the high activation energy barrier (134 kJ/mole) to the release of superoxide from oxyhemoglobin. Therefore, reaction (2) is pulled by reaction (4) and GHP is in effect a methemoglobin superoxide transferase. However, GHP Compound II, that is, GHP(·OH), can use its bound hydroxyl radical (·OH) to oxidize oxyhemoglobin or deoxyhemoglobin to methemoglobin. This scheme also implies that acidification would favor oxidation of hemoglobin mediated by GHP and that alkaline conditions would favor superoxide transfer. Since GHP is an anionic "perioxidase", the development of superoxide from oxygen and an electron on the enzyme would yield an unstable anion-anion interactive intermediate. The repulsive force of the anion-anion interaction would rectify the electric field effect of microwave radiation, resisting superoxide and GHP binding and coupling positively to their dissociation. The opposite effect would be observed on the binding of superoxide to the cationic methemoglobin. The balance between GHP oxidation and reduction of hemoglobin depends on the concentration of H_2O_2 and reductants in the erythrocytes. The concentrations of these reactants, in turn, depend on such enzymes as superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, cytochrome b_5 and its reductase, and glucose-6-phosphate dehydrogenase.

We suggest that the inherent instability of GHP($\cdot O_2^-$) and its destruction of its own heme prevents extensive oxidation of hemoglobin under most adverse conditions and preserves its dominant function as methemoglobin superoxide transferase. However, the thermal susceptibility of the peroxy form of GHP to superoxide release and oxidative destruction suggests that it may drive oxyhemoglobin auto-oxidation during hyperthermia by lowering the activation energy for the reverse methemoglobin superoxide transferase reaction. Finally, the inherent instability of microwave radiation inhibition of oxyhemoglobin auto-oxidation parallels the instability of reduced oxygenated GHP, making it a prime candidate for mediating the thermally-driven and microwave inhibited oxyhemoglobin auto-oxidation. These findings may con-

tribute to better strategies for the long-term storage of blood.

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